

Synthetic Biology of Novel Thermophilic Bacteria for Enhanced Production of Ethanol from 5-Carbon Sugars

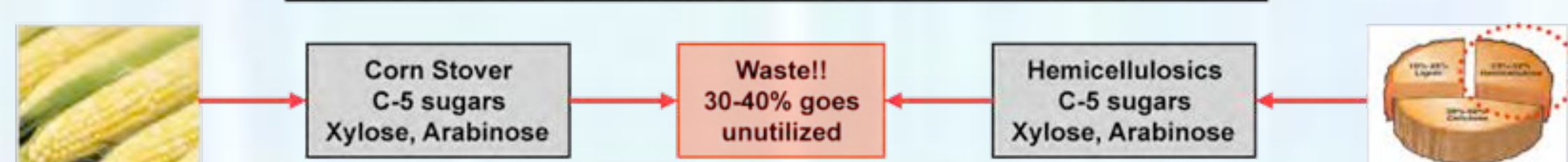


Sandia National Laboratories
Rajat Sapra, Dave Reichmuth, Carrie Kozina, Ken Sale,
Yinjie Tang, Jay Keasling, Harvey Blanch

Problem

**The Problem: Energy Independence.
Part of The Energy Solution ... Plant Biomass**

Bioethanol And Bottlenecks with State of the Art



- Optimal temperature for enzymatic saccharification is 50-60 °C, optimal temperature for most ethanol fermentations is 30 °C

Solution: Simultaneous Saccharification and Fermentation (SSF)

- Conventional fermentation organisms (Yeast) cannot process five-carbon sugars, reducing efficiency and increasing chance of process contamination.

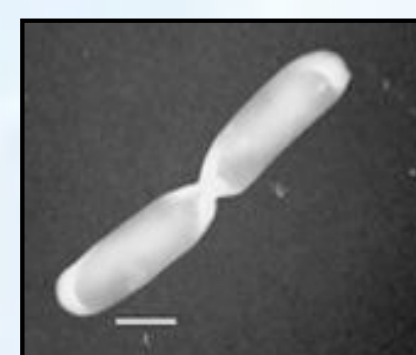
Solution: Organisms that utilize wide range of sugars for ethanol production

Our Proposed Solution: Engineer Thermophilic Bacteria for Ethanol Production

- Geobacillus thermoglucosidarius* M10EXG grows optimally at ~60 °C, is tolerant to 10% ethanol (v/v) and can grow in aerobic and anaerobic conditions
- All other known thermophilic bacteria are tolerant to <4% (v/v) ethanol

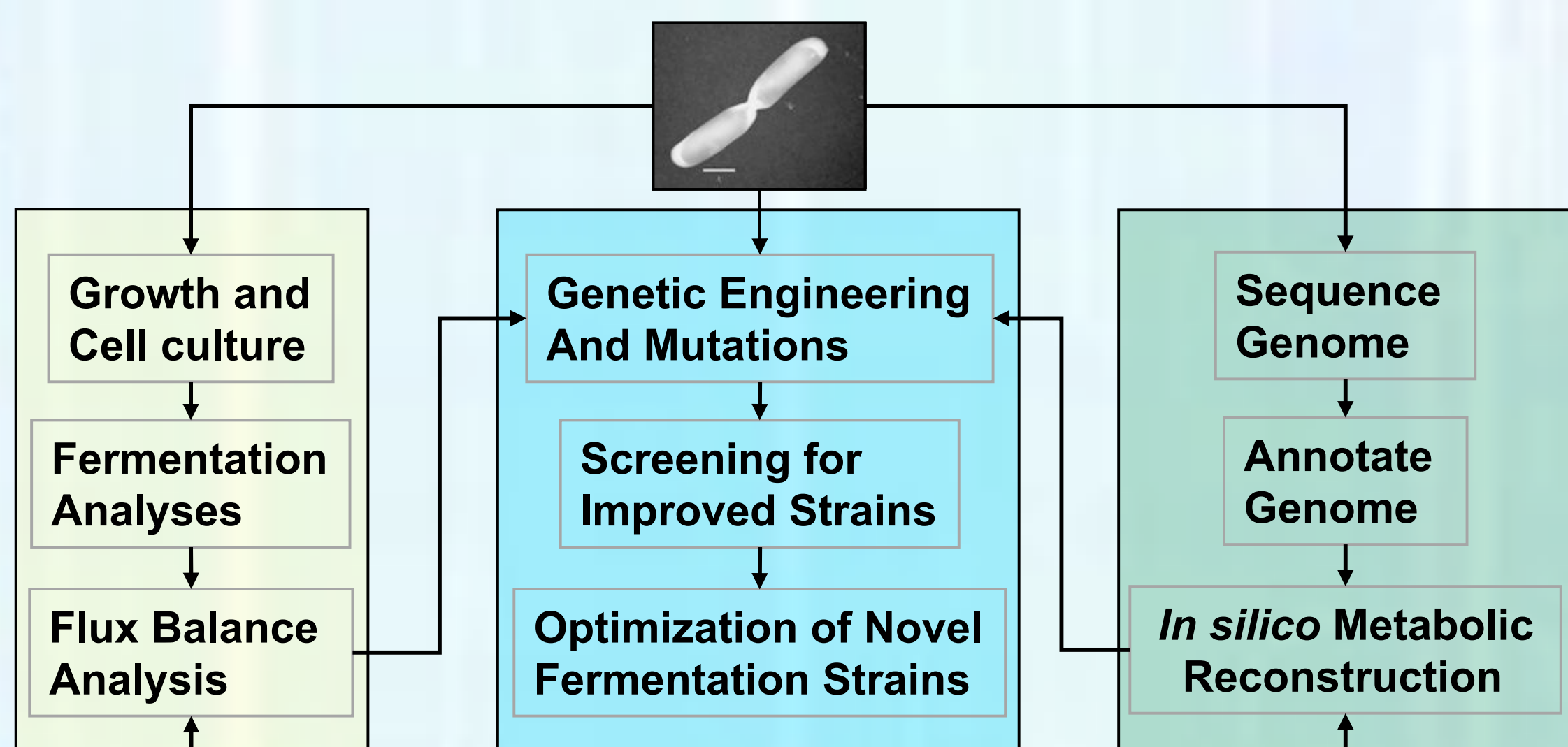
- Produces ethanol, acetate, lactate, from five and six-carbon sugars

- Excellent candidate for SSF if engineered to overproduce ethanol



Approach

Synthetic Biology: Interdisciplinary Approach to Engineering Biological Systems



- G. thermoglucosidarius* (Gth) M10EXG ferments wide range of sugars C5 sugars (xylose)
- Fermentation enzyme already present in Gth M10EXG
- Metabolic engineering of bacterial pathways is currently employed for production of preferred molecules (Keasling, Nature 2006)
- Gth M10EXG has the necessary prerequisites for metabolic engineering
- Engineering thermotolerance in a whole organism is difficult (~impossible)
- Gth M10EXG grown optimally at 60 °C
- Ethanol tolerance is dependent on multiple parameters — nature of membranes, membrane pumps for ethanol, enzymes and membrane proteins.
- Gth M10EXG has the highest tolerance for ethanol among all known thermophilic bacteria

Significance

- Development of bacterial fermentation at high temperatures. Yeast cannot ferment C-5 sugars while bacteria can utilize C5 and C6 sugars.
- High temperature fermentations are compatible with and ideal for Simultaneous Saccharification and Fermentation (SSF) and Consolidated Bioprocessing (CBP) for fuel production & recovery from biomass.
- Improvement in the conversion and recovery of lignocellulose biomass to ethanol.
- Engineered thermophilic platform can be used for development of second-generation hydrocarbon biofuels

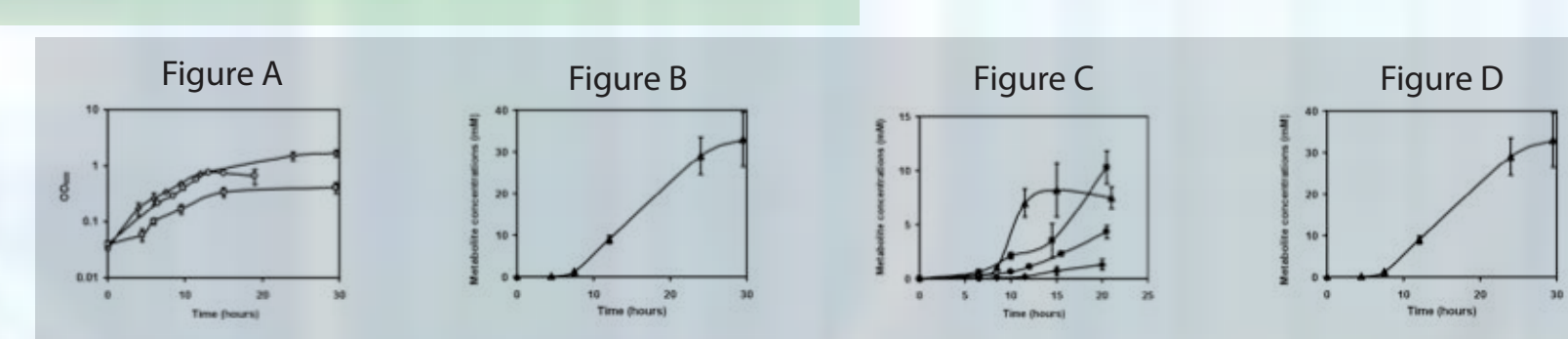
Results (cont.)

Growth kinetics and yield of *Geobacillus thermoglucosidarius* M10EXG under three oxygen conditions (+O₂: aerobic growth, + μO₂: micro-aerobic growth, -O₂: anaerobic growth).

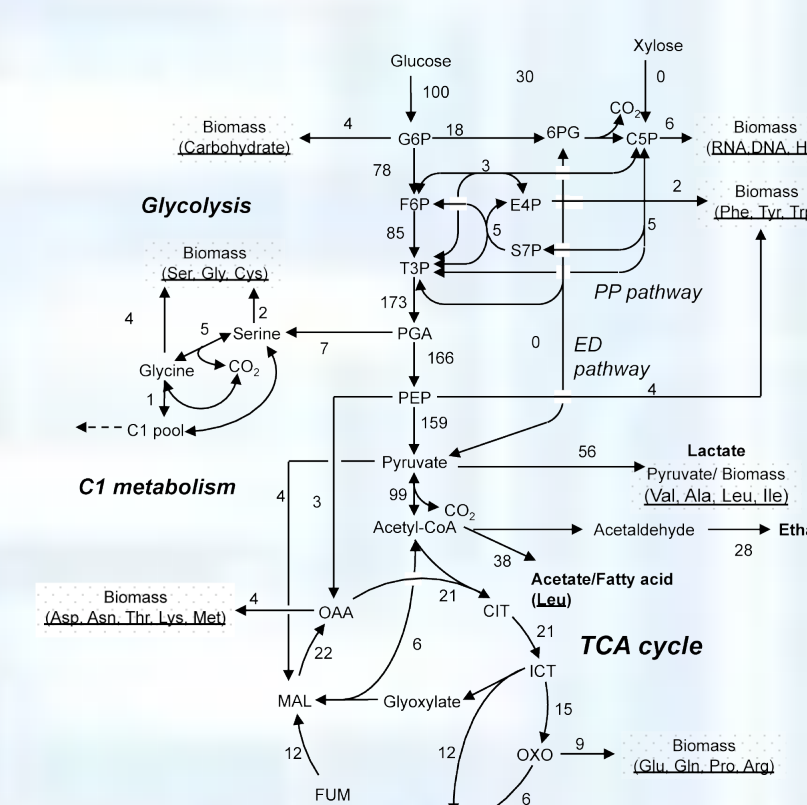
Yield ¹	G + O ₂	G + μO ₂	G - O ₂	Max ²
Y _{glucose}	0.64±0.12	0.40±0.05	0.62±0.11	2.6
Y _{lactate}	0.02±0.01	0.67±0.07	0.81±0.07	2
Y _{ethanol}	0.01±0.01	0.28±0.04	0.32±0.05	2
Y _{formate}	0	0.13±0.05	1.03±0.15	5.6
Y _{acetate}	0.27±0.05	0.19±0.04	0.08±0.03	0.34
Growth rate, hr ⁻¹	0.31±0.04	0.20±0.04	0.13±0.03	0.44

- Metabolite yield unit, mol metabolites mol⁻¹ glucose. Biomass yield unit, g biomass g⁻¹ glucose.
- Small amount of succinate was also detected.
- Max yield for each metabolite was predicted by single optimization using Simpheny. The model assumed the glucose uptake rate equal to 5mM hr⁻¹g⁻¹ biomass.

Biomass growth. Fig A (○: aerobic; □: micro-aerobic; △: anaerobic); Extra-cellular metabolite production. Fig. B: aerobic; Fig. C: micro-aerobic; Fig. D: anaerobic (▲: acetate; ■: Lactate; ★: formic acid; ●: ethanol).



Flux Balance Analysis of Glucose Metabolism Under Micro-aerobic Growth by *G. thermoglucosidarius* M10EXG.

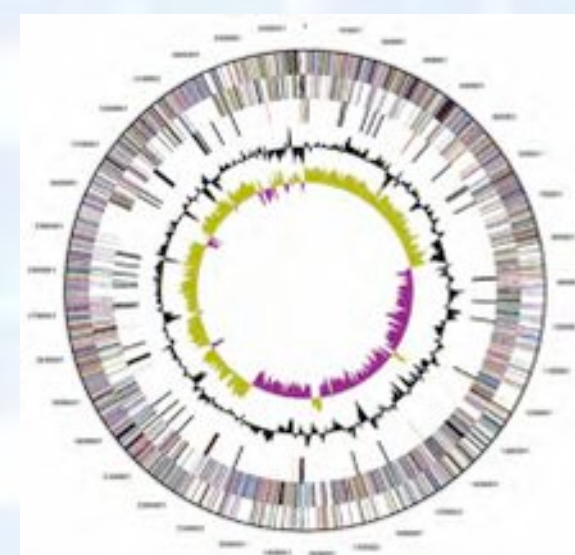


AcCoA, acetyl-coenzyme A;
CIT, citrate;
E4P, erythrose-4-phosphate;
C1, 5,10-Me-THF;
F6P, fructose-6-phosphate;
G6P, glucose-6-phosphate;
6PG, 6-phosphogluconate;
ICT, isocitrate;
MAL, malate;

OAA, oxaloacetate; OXO, 2-oxoglutarate;
PEP, phosphoenolpyruvate;
PGA, 3-phosphoglycerate;
C5P, ribose-5-phosphate (or ribulose-5-phosphate or xylulose-5-phosphate);
S7P, sedoheptulose-7-phosphate; SUC, succinate;
T3P, triose-3-phosphate;
PYR, pyruvate.

The pathway information gleaned will help us better design synthetic pathways

G. thermoglucosidarius M10EXG Chromosomal Map

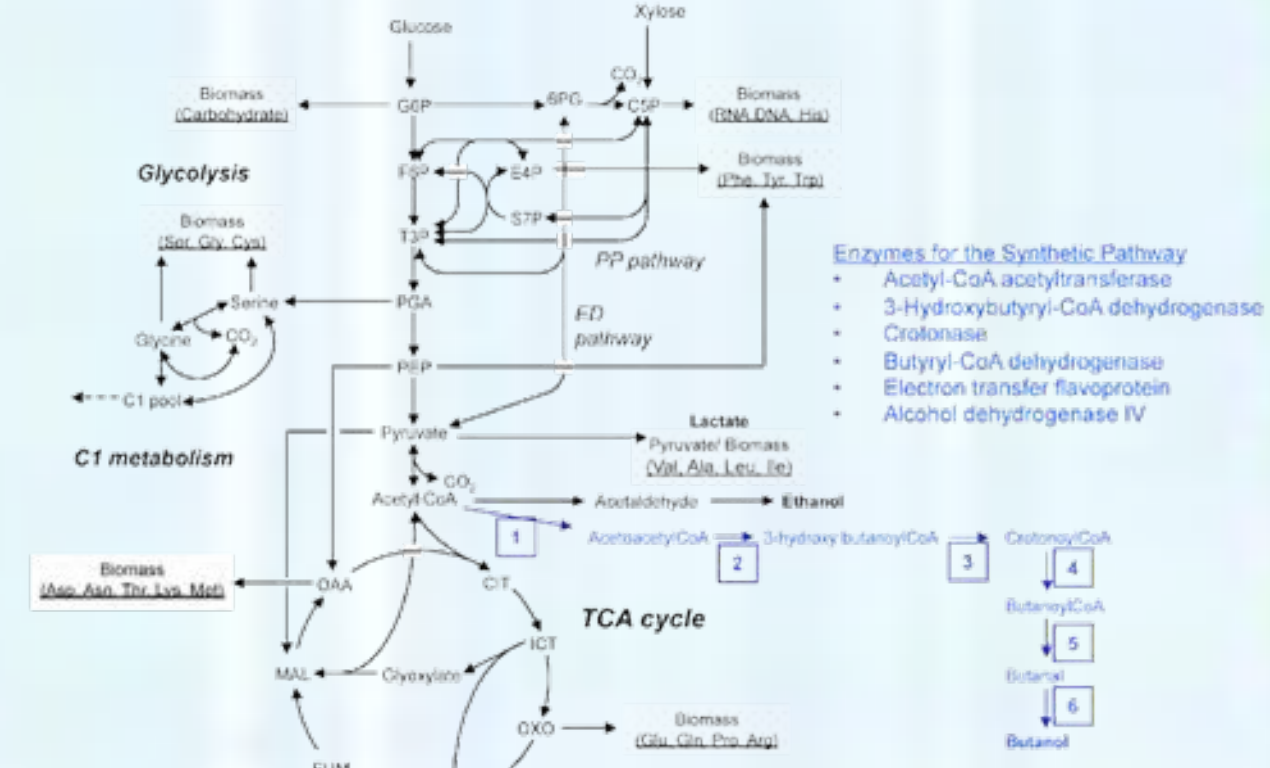


Chromosomal map of genes (From outside of the circular chromosome to the center)
1. Genes on forward strand (color by COG categories), 2. Genes on reverse strand (color by COG categories), 3. RNA genes (tRNAs green, sRNAs red, other RNAs black) 4. GC content 5. GC skew

COG Code	COG Function Definition
[A]	RNA processing and modification
[B]	Chromatin structure and dynamics
[C]	Energy production and conversion
[D]	Cell cycle control, cell division, chromosome partitioning
[E]	Amino acid transport and metabolism
[F]	Nucleotide transport and metabolism
[G]	Carbohydrate transport and metabolism
[H]	Coenzyme transport and metabolism
[I]	Lipid transport and metabolism
[J]	Translation, ribosomal structure and biogenesis
[K]	Transcription
[L]	Replication, recombination and repair

[M]	Cell wall/membrane/envelope biogenesis
[N]	Cell motility
[O]	Posttranslational modification, protein turnover, chaperones
[P]	Inorganic ion transport and metabolism
[Q]	Secondary metabolites biosynthesis, transport and catabolism
[R]	General function prediction only
[S]	Function unknown
[T]	Signal transduction mechanisms
[U]	Intracellular trafficking, secretion, and vesicular transport
[V]	Defense mechanisms
[W]	Extracellular structures
[Y]	Nuclear structure
[Z]	Cytoskeleton

Fuel Map: Engineering Butanol Production Pathway



- Project goals and approach fits well with the DOE mission and program for 5/10/15 year goals:
- '...SSF in thermophilic bacteria for C5 and C6 sugars.'
- Development of cutting edge methods and protocols
- ¹³C isotopomer analysis for FBA is a state-of-the-art HTP method of flux pathways.
- Pathway engineering in thermophilic microorganisms- new genetic tools.
- Metabolic and Kinetic Modeling to rationally engineer flux towards ethanol production